Example 3

Enhanced Protease Resistance

A pulse-chase assay was performed with the strains *B. subtilis* 168 (pLATIL3-BStag) and *B. subtilis* 168 (pLATIL3-DDtag) (Figure 2). NB: the h-IL3 variants expressed by these two strains (hIL3-AA and hIL3-DD) differ only in the last two COOH-terminal amino acids: two alanine residues in hIL3-AA and two aspartic acid residues for hIL3-DD. In the pulse-chase experiment, the initial level (pulse 1 min; chase 0') of extracellular hIL3-DD proved to be roughly 5 times higher than that of hIL3-AA (compare lane 1 with lane 7). In addition, hIL3-AA proteins were degraded with half lives of < 2 min, while the h-IL3-DD molecules had half-lives of approximately 5 min.

Protein labeling, SDS-PAGE, and fluorography. Pulse-chase labeling of B. subtilis and SDS-PAGE was essentially as described previously (Van Dijl et al. 1991. Non-functional expression of Escherichia coli signal peptidase I in Bacillus subtilis. J. Gen. Microbiol. 137:2073-2083). However, samples collected after chase times of 0, 5, 10, 30, and 60 min were centrifuged for 10 seconds, and only the extracellular proteins (in the culture supernatant) were precipitated with trichloroacetic acid (TCA) and eventually subjected to SDS-PAGE. Fluorography was performed with Amplify fluorographic reagent (Amersham-Pharmacia Biotech). Protein bands were quantified using the Storm PhosphorImager system (Molecular Dynamics).

Western blot analysis. To obtain anti-BsSsrAtag antibodies (antibodies that recognize proteins with a C-terminal B. subtilis SsrA-tag), synthetic peptide AGKTNSFNQNVALAA (SEQ ID NO:___) (coupled via an amino-terminal cysteine residue to KLH carrier) was injected into rabbits (Eurogentec). Serum of the final bleed of one of the rabbits was selected for affinity purification, and this purified serum was used in the Western blot procedures. Antibodies against human IL-3 were mouse monoclonals (Van Leen et al. 1991. Production of human interleukin-3 using industrial

microorganisms. Biotechnology 9:47-52). Immunoblotting and detection was performed with alkaline phosphatase-labeled conjugate and the BM Chromogenic Western Blotting kit (Roche Diagnostics) according to the instructions of the manufacturer.

Stability of hIL-3 variants with different C-terminal tags produced by *B. subtilis*. To further investigate whether the *B. subtilis* SsrA tag functions as a degradation signal for secreted proteins, three variants of plasmid pLATIL3 were created. Plasmid pLATIL3BStag contains a gene variant encoding hIL-3 fused at the C-terminus to the *B. subtilis* SsrA peptide tag (AGKTNSFNQNVALAA), plasmid pLATIL3ECtag contains a gene variant encoding h-IL3 fused at the C-terminus to the *E. coli* SsrA tag (AANDENYALAA). The third plasmid pLATIL3DDtag contains a gene encoding h-IL3 fused at the C-terminus to the sequence encoding a DD-tag (AGKTNSFNQNVAL**DD**). This tag is equal to the *B. subtilis* SsrA-tag (AA-tag), but instead of two alanines at the extreme C-terminus it contains two aspartic acid residues. The DD-tag was suspected to be relatively resistant to proteolytic degradation, as observed for *E. coli* (Abo et al. 2000. SsrA-mediated tagging and proteolysis of LacI and its role in the regulation of *lac* operon. EMBO J. 19:3762-3769; Roche et al. 1999). The extracellular proteins produced by cells of *B. subtilis* 168 containing pLATIL3, pLATIL3BStag, pLATIL3DDtag, or pLATIL3ECtag, were analyzed by Western blotting (Fig. 3A). The amount of the hIL-3-DDtag present in the medium was found to be roughly 5 times higher then that of wild-type hIL-3, hIL-3-AAtag or hIL-3-ECtag. Human interleukin-3 molecules produced by wild-type *B. subtilis* are relatively unstable due to proteolytic degradation, and the results represented in figure 3A suggest that addition of a C-terminal SsrA-tag does not lead to increased degradation of hIL-3 molecules. It is important to note, however, that in *E. coli* proteins tagged cotranslationally by the SsrA system are degraded more rapidly than proteins with essentially the same sequence in which the SsrA tag is DNA encoded (Gottesman et al. 1998. The ClpXP

and ClpAP proteases degrade proteins with carboxy-terminal peptide tails added by the SsrA-tagging system. Genes Dev. 12:1338-1347). The results obtained with pLATIL3TERM (Fig. 2) indicate that this is also true for *B. subtilis*. Strikingly, addition of the DD-tag (with two charged, polar residues at the extreme C-terminus) leads to a higher level of extracellular hIL-3, indicating that DD-tagged hIL-3 is less susceptible to proteolytic degradation. To explore this further, a pulse-chase assay was performed with the *B. subtilis* strain 168 (pLATIL3BStag) and 168 (pLATIL3DDtag) (Fig. 3B and 3C). The initial level (chase time = 0 min) of hIL-3-DDtag in the medium is approximately 4 times higher then that of hIL-3-AAtag. In addition, the hIL-3-AAtag variant was degraded with a half-life of < 2 min, whereas the half-life of hIL-3-DDtag was somewhat increased (approximately 5 min). The latter observation supports that DD-tagged hIL-3 is less susceptible to extracellular proteases compared to hIL-3 with an AA-tag. However, the observation that the initial level of hIL3-DDtag in the medium is considerably higher then that of hIL3-AAtag indicates that hIL3-AAtag is also subject to proteolytic degradation before the molecules reach the medium, e.g. during passage of the cell wall of *B. subtilis*.

Example 4

Prolonged Half-life

Detection of cotranslationally SsrA-tagged hIL-3 secreted by WB600 and by cells expressing an SsrA^{DD} variant. To detect SsrA-tagged h-IL3 molecules secreted by *B. subtilis* and to identify proteases that have a role in the degradation of SsrA-tagged hIL-3, two different approaches were used. First, pLATIL3TERM was expressed in WB600, a *B. subtilis* strain lacking six extracellular proteases (Wu et al. 1991. Engineering a *Bacillus subtilis* expression-secretion system with a strain deficient in six extracellular proteases. J. Bacteriol. 173:4952-4958), which may be responsible for the degradation of extracellular, SsrA-tagged hIL-3. In the medium of a culture of WB600 (pLATIL3TERM), a band was detected reacting with antibodies